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TNO report

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Feasibility of screening for antibiotic resistance – part I

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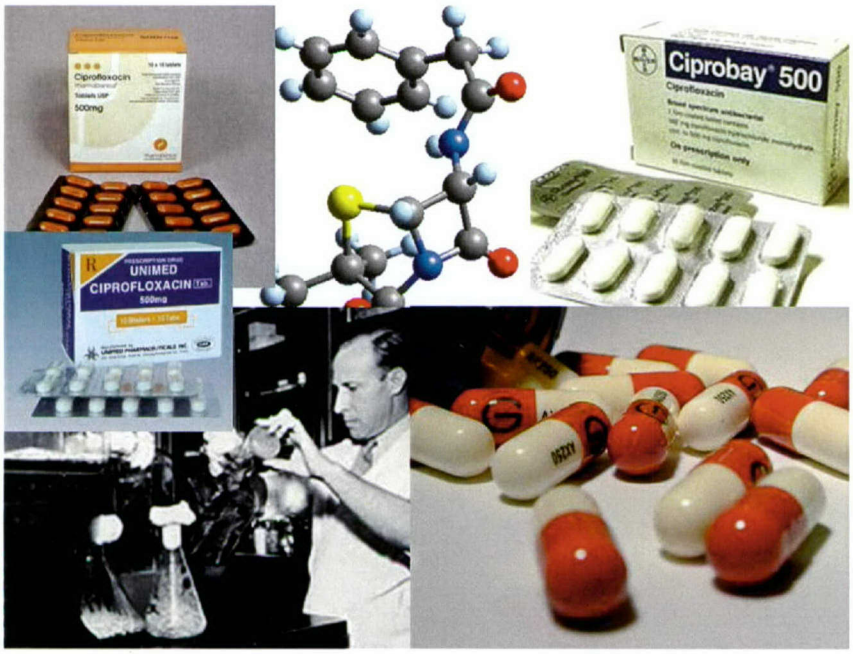
Haalbaarheid van screening op antibiotica-resistentie – deel I



Probleemstelling

Bacteriële infecties veroorzaakt door het gebruik van biologische strijdmiddelen, zijn veelal te bestrijden met antibiotica. Door spontane mutaties (Yersinia pestis in Madagascar) of opzettelijke mutaties (Biopreparat conglomeraat in de voormalige Sovjet-Unie) kan het voorkomen dat een bacteriestam resistent is geworden voor het antibioticum van eerste keus. Hierdoor zal een bacteriële infectie bij de eerste antibioticumkuur niet verminderen, en kan pas bestreden worden door een volgend antibioticum toe te passen. Hiermee gaat kostbare tijd verloren. Een specifiek kenmerk van antibioticaresistente bacteriën is de aanwezigheid van bepaalde resistentiegenen (specifiek erfelijk materiaal) of mutaties (veranderingen) in essentiële genen. Een methode waarmee men snel en rechtstreeks naar specifieke resistentiegenen of mutaties kan zoeken, is Polymerase Chain Reaction (PCR), eventueel in combinatie met het bepalen van de DNA-sequentie. Het doel van de studie is het nagaan of het haalbaar is om op DNA gebaseerde methoden te gebruiken voor het opsporen van antibioticaresistentie. Deze aanpak werd bij TNO Defensie en Veiligheid, locatie Rijswijk, onderzocht in het kader van het Programma V013 'Passieve bescherming tegen NBC-wapens' (resultaatnummer 807a). Het Ministerie van Defensie is de opdrachtgever van dit Programma.

Beschrijving van de werkzaamheden

Nagegaan werd in hoeverre een PCR-methode kon worden ontwikkeld voor het aantonen van bekende resistentiegenen die zijn gerelateerd aan resistentie van bacteriën tegen de antibiotica ampicilline en/of tetracycline, respectievelijk het Ap-R en het Tc-R gen. Daarnaast werd een aantal 'reactieomstandigheden' in de PCR-assay geoptimaliseerd. Verificatie van het gerepliceerde DNA was mogelijk door middel van gel-electroforese.
Tevens werd de assay gevalideerd door het aantonen van de genen na transformatie van naïeve stammen met de DNA-klonen. Hierbij worden de resistentiegenen ingebouwd in bacteriën waarbij die genen van nature niet aanwezig zijn.
Een begin werd gemaakt met het opzetten van een assay om in een bacterie mutaties in de genen gyrase (gyrA) en topoisomerase IV (parC) op te sporen. Dit betrof mutaties die zijn geassocieerd met resistentie tegen ciprofloxacine. Ciprofloxacine is een breed-spectrum antibioticum en de eerste keus voor behandeling van o.a. anthrax.

Resultaten en conclusies

Het bleek mogelijk een PCR-methode op te zetten voor het aantonen van de genoemde resistentiegenen tegen ampicilline en

tetracycline. Deze PCR-assays konden worden uitgevoerd met geïsoleerde plasmides en met bacteriesuspensies. Het idee van screenen op antibioticaresistentie bleek derhalve haalbaar te zijn. Vervolgens werd onderzoek gedaan aan ciprofloxacine. De eerste resultaten met deze verbinding zijn eveneens positief. Mutaties in de enzymen gyrA en parC konden worden aangetoond na bepaling van de DNA-sequentie van een PCR-product vanuit een bacteriesuspensie.

Toepasbaarheid

Indien militairen zijn blootgesteld aan een B-wapen, is er behoefte aan een snelle en efficiënte therapie. De identiteit van het agens alsmede de aanwezigheid van specifieke resistentiegenen of mutaties in essentiële genen van het agens kunnen mede bepalend zijn voor de keuze van het therapeuticum. Op basis van goede informatie kan na een besmetting snel een efficiënte therapie worden gestart. Dit zal de inzetbaarheid van militair personeel vergroten.

Vervolgafspraken

Het vervolgonderzoek zal bestaan uit het afronden van onderzoek naar ciprofloxacine (resultaatnummer 807a) en het onderzoek in Programma V013 onder resultaatnummer 807b.

PROGRAMMA	PROJECT
Programmabegeleider A.S. de Koning, CDC/MGFB	Projectbegeleider Dr. H.J. Jansen, MGFB
Programmaleider Dr. Ir. M.S. Nieuwenhuizen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming	Projectleider Dr. M. Polhuijs (in 2001), TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming
Programmatitel Passieve verdediging tegen NBC-wapens	Projecttitel Feasibility screening antibiotica resistentie
Programmanummer V013	Projectnummer 014.14004
Programmaplanning Start 2001 Gereed 2005	Projectplanning Start 2001 Gereed 2002
Frequentie van overleg Met de programma/projectbegeleider werd meerdere malen gesproken over de invulling en de voortgang van het onderzoek.	Projectteam Dr. M. Polhuijs, A.M.B.C. de Vries, W.C.M. van Dijk, Ir. M.P. Broekhuijsen, TNO Defensie en Veiligheid, Business Unit Biologische en Chemische Bescherming

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Summary

Bacterial infections, amongst them those resulting from exposure to biological warfare agents, can be treated with antibiotics. Due to spontaneous mutations (as described for *Yersinia pestis* in Madagascar) or genetic modification of bacterial strains (as might have been performed at the Biopreparat conglomerate of the former Soviet-Union), bacteria can develop resistance against the most commonly used antibiotics. As a result, it might be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time will be lost before a second, more efficient antibiotic compound can be administered. Resistance of bacteria against antibiotics is characterized by the presence of specific resistance genes or specific mutations in genes. The aim of this work is to study the feasibility of developing DNA-based methods that can be used to detect antibiotic resistance in bacteria.

If military personnel are exposed to a biological warfare agent, therapy is needed. In order to start a rapid and efficient therapy, especially in the case of micro-organisms that are resistant to (several) antibiotics, the identity of both the agent and the genes associated with the resistance against antibiotics must be known. A method that provides rapid and unequivocal information on the presence of specific genes is essential. Polymerase Chain Reaction (PCR) is such a method.

First, it was studied whether it was possible to set up a PCR assay for the detection of genes that are associated with resistance against the model compounds ampicillin and tetracycline. Ampicillin resistance can be associated with the presence of the Ap-R gene, ß-lactamase in, for instance, the plasmids pUC18, Bt-PA3 and pBR325. Tetracycline resistance is associated with the presence of the Tc-R gene, tetracycline resistance protein in, for instance, the plasmid pBR325. The presence of these genes, that are normally not present in susceptible bacteria, can be detected with a PCR assay. The presence of these genes could ultimately result in expression of resistance against tetracycline and ampicillin.

In addition, preliminary tests were performed to set up assays to detect mutations in the gyrase (gyrA) and topoisomerase IV (parC) genes of bacteria. These mutations are associated with the antibiotic resistance against the more military relevant antibiotic compound, ciprofloxacin.

From the results obtained in this feasibility study, it can be concluded that it is possible to detect specific plasmid genes associated with resistance to certain antibiotics such as tetracycline and ampicillin in whole bacteria. In addition, it appeared possible to set up methods to detect mutations in essential genes of bacteria. The pattern of the mutations can provide information on the possibility of antibiotic resistance against military relevant antibiotics such as ciprofloxacin.

Detection of specific genes with PCR or detection of mutations in specific genes by means of PCR, followed by DNA sequencing can help to construct the outline of an efficient therapy. This will contribute to fulfilling one of the main objectives of the Ministry of Defense in the area of 'Passive NBC Protection', i.e. 'Protection of personnel against adverse health effects as a result of (non intentional) exposure to biological agents under operational circumstances'

Contents

1 Introduction

Bacterial infections, amongst them those resulting from exposure to biological warfare agents, can be treated with antibiotics. However, because of the wide-spread use of these compounds, resistance of micro-organisms against those antibiotics is becoming a large problem in the clinical setting [Weigel et al., 1998]. As a result, it might be possible that a bacterial infection cannot be treated efficiently with the antibiotic of choice and that precious time will be lost before a second, more efficient antibiotic compound can be administered. Resistance of bacteria against antibiotics is characterized by the presence of specific resistance genes or specific mutations in genes.

Resistant *Yersinia pestis* strains, most likely due to spontaneous mutation, were discovered in Madagascar. In addition, it is assumed that within the Biopreparat conglomerate of the former Soviet-Union, certain bacterial strains were genetically modified in order to become resistant against most of the commonly used antibiotics. In literature, methods for the general approach of modification are well documented. As a result, the use of antibiotic resistant bacteria strains becomes a real threat.

If military personnel are exposed to a biological warfare agent, therapy is needed. In order to start a rapid and efficient therapy, especially in the case of micro-organisms that are resistant to (several) antibiotics, the identity of both the agent and the genes associated with the resistance against antibiotics must be known. A method that provides rapid and unequivocal information on the presence of specific genes is essential. Polymerase Chain Reaction (PCR) is such a method.

First, it was studied whether it was possible to set up a PCR assay for the detection of genes that are associated with resistance against the model compounds ampicillin and tetracycline. The use of these compounds in hospitals is not very frequent because of emerging resistance against these antibiotics. But, due to this phenomenon, the genes associated with the resistance are described in great detail and all material is easily available.
Ampicillin resistance can be associated with the presence of the Ap-R gene, ß-lactamase in, for instance, the plasmids pUC18, Bt-PA3 and pBR325. Tetracycline resistance is associated with the presence of the Tc-R gene, tetracycline resistance protein in, for instance, the plasmid pBR325. The presence of these genes, that are normally not present in susceptible bacteria, can be detected with a PCR assay. The presence of these genes could ultimately result in expression of resistance against tetracycline and ampicillin.
Pending the results with these compounds, it was planned to extend the study to a more military relevant antibiotic compound, ciprofloxacin. Resistance against ciprofloxacin can be associated with mutations in the quinolones resistant determining region (QRDR) in subunits of DNA gyrase (gyrA and gyrB), in the QRDR of subunits of Topoisomerase IV (parC and parE) and an increase in the activity of an efflux pump [Zeller et al., 1997; Balas et al., 1998; Weigel et al., 1998]. In gram negative bacteria, mutations in gyrA are more abundant than in parC. For gram positive bacteria, mutations in parC are more abundant than in gyrA. Since the resistance is associated with mutations in existing genes, the genes must be amplified and the DNA sequence must be determined in order to determine the presence of mutations, indicating the possibility of expression of resistance.

2 Materials and Methods

2.1 Equipment and Software

The PTC200 PCR apparatus from Biozym was used. Gel electrophoresis was performed with agarose gels and equipment from BioRad and power supply from Pharmacia. DNA sequence manipulation, choice of restriction enzymes and primer design were performed with DNAMAN 4.11, Lynnon BioSoft.

2.2 Biological materials

The plasmid Bt-PA3 was a gift from Dr. Netty Zegers, at that time, TNO-PG Leiden. Plasmid pUC18 was from GibcoBRL and pBR325 from Sigma. *Escherichia coli* (11775) was from ATCC. JM109 *Escherichia coli* and XL1-Blue MRF' Kan Super competent Cells were purchased from Stratagene. Bacterial thermolysates (certified killed) of *Y. Pestis* KIM 5, *Y. pseudotuberculosis* NCTC 10275, and *Fransicella tularensis* 237 were kindly provided by dr. Mats Forsman from the Swedish Defence Research Agency (FOI) in Umeå, Sweden. The restriction enzymes Nco I and Apal I were purchased from Eurogentec.

2.3 Chemicals en materials

Two primer pairs for the amplification of the Ap-R gene were from Dr. Martien Broekhuijsen (11031F014/11031R014, amplicon of 350 bp; 11031F015/11031R015, amplicon of 595 bp; Report number PML 2001-B5). The synthetic oligonucleotide primers were obtained from Isogen, Bioscience BV. DNA Molecular Weight Marker 9 was from Eurogentec (Φcut with HinfI, size in bp 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 42, 40, 24. Taq DNA Polymerase was bought from Roche and PCR Nucleotide Mix from Boehringer. Ciprofloxacin (infusion fluid; 200 mg/100 ml) was from Bayer.

2.4 Primer design

2.4.1 Ampicillin

For the primer design in case of the Ap-R gene in pBR325 cloning vector, the sequence-ID code in Genbank L08855 was used (position Ap-R gene 3298-4086). Primer locations were chosen to result in PCR products of between 200 and 300 bp. Amplicons of 200-300 bp were chosen (sense primer 3300-3700 and antisense primer 3500-4000; length 18-25, Tm 60-61 °C, GC 40-60%). Primers were Ap-F04 (5'-TCCATAGTTGCCTGACTCCC-3'),
Ap-R04 (5'-GCAACAATTAATAGACTGGATGGAG-3'),
Ap-F05 (5'-ACGGGAGGGCTTACCATCT-3'),
Ap-R05 (5'-CAAACGACGAGCGTGACA-3'), Ap-F06 (5'-CGTTTGGTATGGCTTCATTCA-3') and Ap-R06 (5'-TGTGGCGCGGTATTATCC-3'). Amplicons of 201, 251 and 298 bp resulted from the primer combinations Ap-F04/Ap-R04, Ap-F05/Ap-R05 and Ap-F06/Ap-R06, respectively.

2.4.2 *Tetracycline*

For the primer design in case of the Tc-R gene in pBR325 cloning vector, the sequence-ID code in Genbank L08855 was used (position Tc-R gene 86-1276). Primer locations were chosen to result in PCR products of between 200 and 300 bp. Amplicons of 200-300 bp were chosen (sense primer 86-700 and antisense primer 200-880; length 18-25, Tm 60-61 °C, GC 40-60%). Primers were
Tc-F01 (5'-GCTTGGTTATGCCGGTACTG-3'),
Tc-R01 (5'-TCGCGTAGTCGATAGTGGCT-3'),
Tc-F02 (5'-GGTTGCTGGCGCCTATATC-3'),
Tc-R02 (5'-TGAAGGCTCTCAAGGGCA-3'),
Tc-F03 (5'-GCTGCTAGCGCTATATGCGT-3') and
Tc-R03 (5'-TACCCACGCCGAAACAAG-3'). Amplicons of 201, 251 and 290 bp resulted from the primer combinations Tc-F01/Tc-R01, Tc-F02/Tc-R02 and Tc-F03/Tc-R03, respectively.

2.4.3 *Ciprofloxacin*

For the primer design in case of the gyrA gene the sequence-ID code in Genbank from various bacteria were used: *Citrobacter freundii* (AF052253), *E. coli* (AF052254), *Enterobacter aerogenes* (AF052255), *Enterobacter cloacae* (AF052256), *Klebsiella oxytoca* (AF052257), *Klebsiella pneumonia* (AF052258), *Providencia stuartii* (AF052259), *Serratia marcescens* (AF052260). After alignment of the sequences of the various QRDR's, primer locations were chosen that would result in the same PCR product when using the various bacteria as a source of DNA. Primers for the enzyme gyrase A were GyrA-F01 (5'-TC(G/C)TATCTGGA(C/T)TATGCGATGT-3'), GyrA-R02 (5'-CGGATTTCCGTATAACGAT-3') and GyrA-R03 (5'-CGAATTTCGGTATAACGCAT-3'). Amplicons of 320 bp resulted from both the primer pairs gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03.
In the case of *F. tularensis* 237, the reversed primer GyrA-R03 could be used, but, another forward primer had to be designed: GyrA-F04 (5'-AATTGTTGGTCGTGCTTTGC-3'). With the primer pair gyrA-F04/gyrA-R03, an amplicon of 294 was produced when using *F. tularensis* 237 as a source of DNA.
For the primer design in case of the parC gene in E. coli, the sequence-ID code in Genbank M58408 was used (position parC gene 92-2284). Primer locations were chosen to result in a PCR product without the amplification of another product from the gyrA gene. Primers for the enzyme par C were
ParC-F01
(5'-GGTGATGGTCTGAAACCTGTTC-3') and ParC-R01
(5'-GCTCGGAATATTTCGACAACC-3'). An amplicon of 292 bp resulted from the primer pair parC-F01/parC-R01.

2.5 Genbank accession numbers

E. coli gyrA (AF052254), *E. coli* parC (M58408), *Y. pestis* gyrA, (AF282314), *Y. pseudotuberculosis* gyrA (AF282315).
The sequence of *F. tularensis* 237 gyrA was obtained from dr. Pär Larsson from the Swedish Defence Research Agency (FOI) in Umeå, Sweden.

2.6 DNA sequence

DNA sequence of amplified material was determined by Base Clear.

2.7 PCR amplicification

PCR amplification was performed with 2.5 U of Taq polymerase, circa 0.75 pg of DNA (plasmid or chromosomal), a 0.2 µM concentration of each synthetic oligonucleotide primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 1.5 mM $MgCl_2$ in a PCR volume of 50 µl. Amplification was started with an initial cycle of 5 min denaturation at 92 °C. Followed by 35 cycles, each consisting of 30 sec denaturation at 92 °C, 30 sec of annealing at 60 °C, and 30 sec of polymerase elongation at 72 °C. After these cycles, temperature was kept at 72 °C during 5 min in order to allow complete elongation of all product DNA, followed by cooling to 15 °C. Amplification products were visualized by agarose gel electrophoresis (1.2% wt/vol agarose) and ethidium bromide staining to confirm the sizes of the products.

2.8 Amplification of the Ap-R gene from the plasmid Bt-PA3

The Bt-PA3 plasmid was directly added to the PCR assay as described above. The Ap-R gene from the plasmid was amplified in the PCR assay with the ampicillin primer pair 11031F014/11031R014.

2.9 Amplification of the Tc-R gene from the plasmids pUC18, Bt-PA3 and pBR325

The pUC18, Bt-PA3 and pBR325 plasmids were directly added to the PCR assay as described above. The Tc-R gene from the plasmids was amplified in the PCR assay with the tetracycline primer pairs Tc-F01/Tc-R01, Tc-F02/Tc-R02 and Tc-F03/Tc-R03.

2.10 Amplification of the Tc-R and the Ap-R gene from bacterial suspension

Transformation of Epicurian XL1-Blue MRF' Kan Super competent cells with pBR325 plasmid, or pUC18 plasmid was performed according to the instructions of the manufacturer. Thereafter, the transformed cells were directly added to the PCR assay as described above. In addition, the Tc-R gene from the pBR325 plasmid was amplified in a separate PCR assay as a positive control. All assays were performed with the tetracycline (Tc-F01/Tc-R01, Tc-F02/Tc-R02 and Tc-F03/Tc-R03) and the ampicillin (Ap-F04/Ap-R04, Ap-F05/Ap-R05 and Ap-F06/Ap-R06) primer pairs.

2.11 Amplification of the gyr A gene in *E. coli*, *Y. pestis*, *Y. pseudotuberculosis* and *Fransicella tularensis*

DNA from bacterial suspension of *E. coli*, *Y. pestis* and *Y. pseudotuberculosis* was amplified in the presence of the primer pairs gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03. PCR amplification was performed as described above.
For *Fransicella tularensis* 237 the primer pair gyrA-F04/gyrA-R03 was used in the PCR assay. This assay was performed with two concentrations of bacteria (one order of magnitude difference). As a validation of the assay, part of the gyrA gene from various strains of *Fransicella tularensis* (strains 021, 022, 032, 040, 046, 080, 148, 155, 230; bacterial thermolysates (certified killed); donated by dr. Mats Forsman from the Swedish Defence Research Agency (FOI) in Umeå, Sweden) were amplified as well. The sequence of these amplicons were determined.

2.12 Experiments with restriction enzymes

The amplified PCR product of assays in which *E. coli* XL1 blue was used as a source of DNA and the primer pair gyrA-F01/gyrA-R02, was incubated with the restriction enzyme NC01. This enzyme should cut the amplicon of 320 bp in two smaller products of 180 and 140 bp. In the case of *Y. pestis* and *Y. pseudotuberculosis*, the primer pair gyrA-F01/gyrA-R03 was used, also resulting in an amplicon of 320 bp. The restriction enzyme ApaI1 was used in order to get two new products of 247 and 73 bp.

2.13 Amplification of the gyrA and the parC gene from *E. coli*

In another experiment, parts of the the gyrA and the parC gene from *E. coli* were amplified with the primer pairs gyrA-F01/gyrA-R02 and parC-F01/parC-R01, respectively. *E. coli* strains used were the wild type strain ATCC 11775 (K12 origin), XL1-Blue and JM109 (no K12 origin). Amplicons of the gyrA (320 bp) and parC (292) were detected as described above. The sequence of all PCR products was determined.

3 Results

3.1 Amplification of the Ap-R gene from the plasmid Bt-PA3

When using the primer pair 11031F014/11031R014, it was possible to amplify a product when using the method as described in Paragraph 2.7 'PCR amplification'. A distinct amplicon of 350 bp was present in the assays where plasmid Bt-PA3 was used as a source of DNA (Figure 1, lanes 4, 5, 9 and 10). For the lanes 4 and 9, the amount of primer pair was twice that of the assays from lanes 5 and 10. But in both assays, the desired product was formed. With higher amounts of primer pairs no byproducts or primer-dimer products were observed. When no DNA was present in the assay, no product was formed.

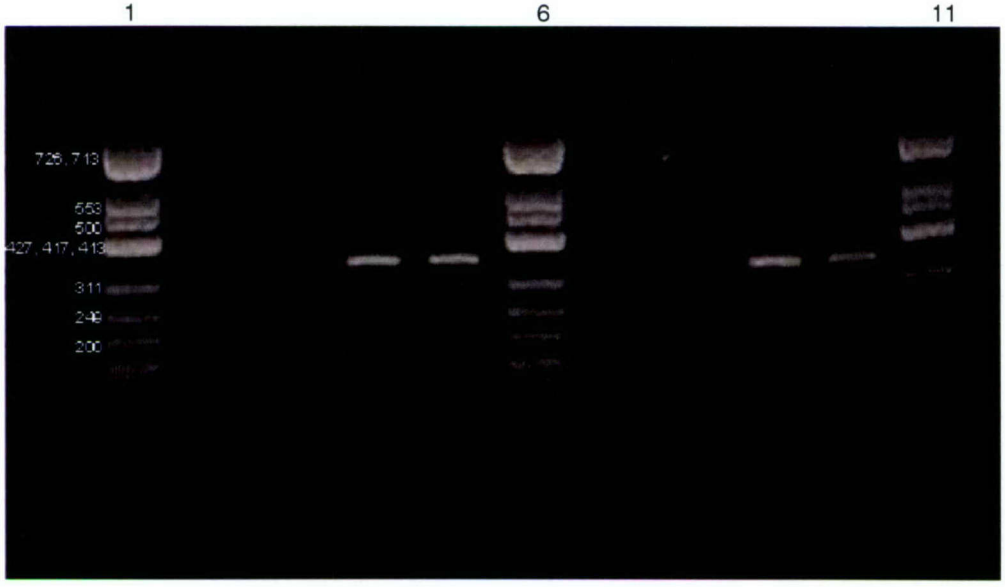

Figure 1 The ladder used is marker M9 (lanes 1, 6 and 11). In lanes 4, 5, 9 and 10, 0. 75 pg DNA was used. Lanes 2, 3, 7 and 8 were blank controls with MQ water. In lanes 2, 4, 7 and 9, twice the regular amount of primer was added.

3.2 Amplification of the Tc-R gene from the plasmids pUC18, Bt-PA3 and pBR325

DNA from the three plasmids was incubated with the three different primer pairs for amplification of the Tc-R gene. With each primer pair, a distinct amplicon was produced which corresponded with the anticipated molecular weight as visualized in Figure 2. The primer pair Tc-F03/Tc-R03, resulted in an amplicon of 290 bp (lane 8 and 9). For Tc-F02/Tc-R02 this was an amplicon of 251 bp (lane 14) and for Tc-F01/Tc-R01 this was an amplicon of 201 bp (lane 19). Therefore, all three primer pairs could be used to amplify the Tc-R gene in the three plasmids studied. When no DNA was added, no products were detected (lane 5, 11 and 16).

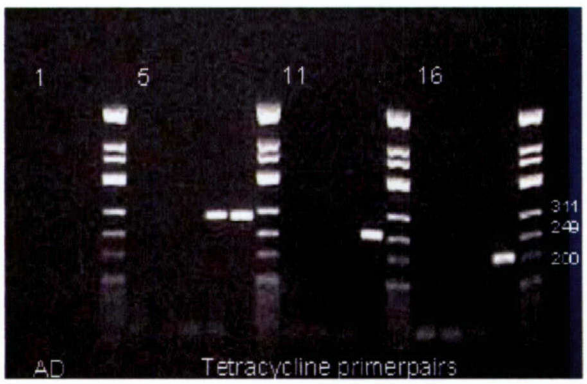

Figure 2 The ladder used is marker M9 (lanes 4, 10, 15 and 20). In lane 1, 6, 12 and 17 the plasmid pUC18 was used as a source of DNA (0.75 pg). In lane 2, 7, 13 and 18 this was the plasmid Bt-PA3 and in lane 3, 8 (less DNA than in lane 9), 9, 14 and 19 the plasmid pBR325. The lanes 5, 11 and 16 are blank controls where no DNA was added. No primer pairs were added to the assays belonging to the lanes 1, 2 and 3. In the lanes 5-9, the primer pair Tc-F03/Tc-R03, resulting in an amplicon of 290 bp, was used. For the lanes 11-14 this was Tc-F02/Tc-R02 (amplicon of 251 bp) and for the lanes 16-19 this was Tc-F01/Tc-R01 (amplicon of 201 bp).

3.3 Amplification of the Tc-R and Ap-R genes in bacterial suspension

Epicurian XL1-Blue MRF' Kan Super competent cells were transformed with the plasmids pBR325, containing both the Tc-R and the Ap-R genes, and pUC18, containing the Ap-R gene. The transformed Epicurian XL1-Blue MRF' Kan Super competent cells were used in the PCR assays without DNA isolation in the presence of the three different ampicillin and tetracycline primer pairs.
When using the ampicillin primer pairs Ap-F04/Ap-R04, Ap-F05/Ap-R05 and Ap-F06/Ap-R06, the expected amplicons are 201 bp, 251 bp and 298 bp, resp. For the tetracycline primer pairs Tc-F01/Tc-R01, Tc-F02/Tc-R02 and Tc-F03/Tc-R03, the resulting amplicons are 201 bp, 251 bp and 290 bp. The pBR325 plasmid, containing both the Tc-R and the Ap-R genes, was used in the PCR assay as a positive control.
In the assays where the pBR325 plasmid as such (Figure 3, lane 2-7) or the transformed cells containing this plasmid (Figure 3, lanes 9-14) were used as a source of DNA, amplicons corresponding with the various ampicillin and tetracycline primer pairs could be detected.
In the lanes 15, 16 and 17 of Figure 3, where pUC18 was used as a source of DNA, no amplified product could be detected. In the corresponding PCR assays, the three tetracycline primer pairs were used. Since pUC18 does not possess the Tc-R gene, there should be no amplified product in these lanes. These assays were intended to serve as a negative control. Indeed, no product could be detected. In addition, in blank control assays where no DNA was added, no product could be detected (data not shown). The various ampicillin primer pairs were able to amplify the Ap-R gene of the transformed cells containing the pUC18 plasmid, as based on the formation of the three different products in lanes 18, 19 and 20 (Figure 3).

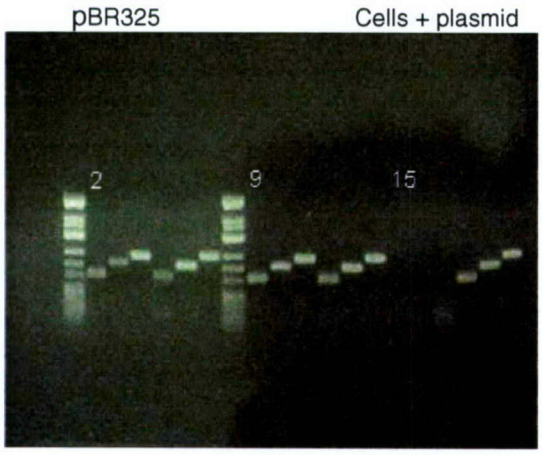

Figure 3 The ladder used is marker M9 (lanes 1 and 8). In lanes 2-7, the plasmid pBR325 was used as a source of DNA. In the lanes 9-14, transformed pBR325 was used as a source for DNA. In the lanes 15-20, transformed pUC18 was added to the PCR assay. In the lanes 2, 9 and 15, the primer pair Tc-F01/Tc-R01, resulting in an amplicon of 201 bp when DNA was present, was used. For the lanes 3, 10 and 16 this was Tc-F02/Tc-R02 (amplicon of 251 bp in the case of available DNA) and for the lanes 4, 11 and 17 this was Tc-F03/Tc-R03 (amplicon of 290 bp in the case of available DNA). The primer pair Ap-F04/Ap-R04, resulting in an amplicon of 201 bp, was used in lane 5, 12 and 18. The amplicon of 251 bp in lanes 6, 13 and 19 resulted from the use of the primer pair Ap-F05/Ap-R05 and that of 298 bp in lanes 7, 14 and 20 from the use of the primer pair Ap-F06/Ap-R06.

3.4 Selective amplification of a gyrA fragment in bacterial suspension

Oligonucleotide primers selected from the conserved gyrA gene sequences flanking the QRDR were developed. It was anticipated that two of the developed primer pairs, gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03, would be able to amplify the gyrA fragment from chromosomal DNA present in the species *E. coli*, *Y. pestis* as well as *Y. pseudotuberculosis*.
This appeared to be successful for the two primer pairs in the case of the *Y.* species (Figure 4, lanes 5-8).

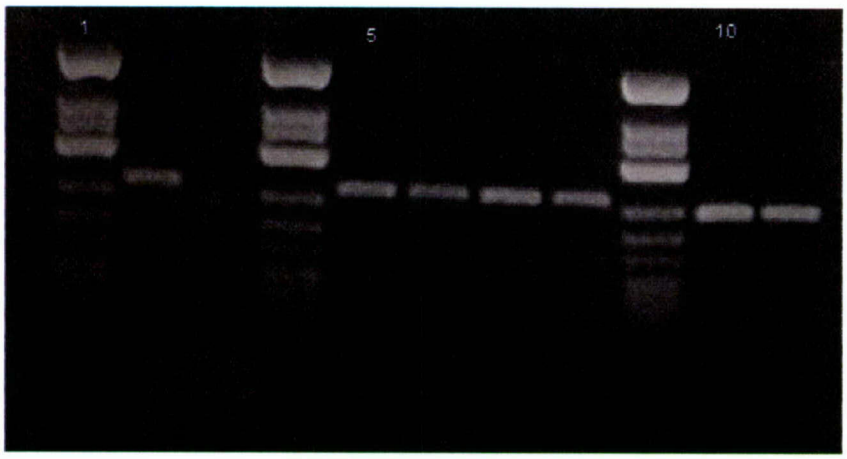

Figure 4 MW-marker is M9 (lane 1,4 en 9; 726/713, 553, 500, 427-413, 311, 249, 200, 151, 140, 118, 100-24). The various bacteria used were *E. coli* (lane 2, 3), *Y. pestis* (lane 5, 6), *Y. pseudotuberculosis* (lane 7, 8) and *F. tularensis* (lane 10, 11). Primer pair gyrA-F01/gyrA-R02 was used in lane 2, 5 and 7. For lane 3, 6 and 8 this was gyrA-F01/gyrA-R03. In lane 10 and 11 (DNA in this assay was one order of magnitude lower than in the assay from lane 10), primer pair gyrA-F04/gyrA-R03 was used.

Notwithstanding the fact that the binding site of the primer pairs for the gyrA and the parC are highly homologous, one amplicon was produced when using one primer pair. This was determined by experiments in which amplified products were split into two distinct parts by restriction enzymes. The amplicon of 320 bp produced with the primer pair gyrA-F01/gyrA-R03 and *Y. pestis* and *Y. pseudotuberculosis* as a source of DNA, was digested in two smaller products of 247 and 73 bp after incubation with the restriction enzyme ApaI1 (Figure 5, lanes 3 and 4, respectively). The product of 247 bp is clearly visible. The 73 bp fragment was just visible on the original gel, but not on the photo that was made thereof. Most likely, the amount of DNA was too low for the quality of the camera used. In addition, some remainder of the 320 bp product was found. No other products could be visualized. To test whether two products were amplified, the product in a sample from the another PCR assay was sequenced. Only one product was found and its sequence corresponded with the sequence of the gyrA fragment of *Y. pestis* and *Y. pseudotuberculosis* as found in literature.

When *E. coli* was used as a source of DNA, the use of primer pair gyrA-F01/gyrA-R02 resulted in the production of the expected amplicon (Figure 4, lane 2). However, the primer pair gyrA-F01/gyrA-R03 was not able to amplify the gyrA fragment (Figure 4, lane 3), indicating that the sequence of the gyrA fragment in this organism diverges from the conserved sequences shared by the Y. species. The amplicon of 320 bp produced with the primer pair gyrA-F01/gyrA-R02 and *E. coli* as a source of DNA, was digested in two smaller products of 180 and 140 bp after incubation with the restriction enzyme NC01 (Figure 5, lane 2). In addition, some remainder of the 320 bp product was found. No other products could be visualized. To test whether two products were amplified, the product in a sample from the same PCR assay that was not incubated with restriction enzyme was sequenced. Only one product was found and its sequence corresponded with the sequence of the gyrA fragment as found in literature.
Based on the sequence of the conserved sequences flanking the QRDR of *F. tularensis*, it was anticipated that the primer pairs gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03 would not be able to amplify the gyrA fragment from this species. Therefore, another primer pair was developed, gyrA-F04/gyrA-R03. This primer pair appeared to be able to produce the expected amplicon with a lower (Figure 4, lane 11) or a higher amount (Figure 4, lane 10) of DNA in the PCR assay.

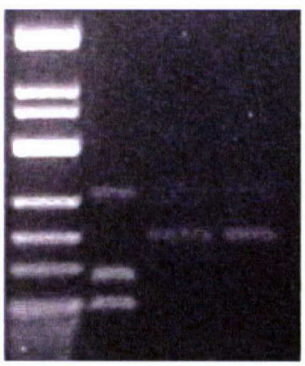

Figure 5 MW-marker is M9 (lane 1). The various bacteria used were *E. coli* (lane 2, gyrA-F01/gyrA-R02; restriction enzyme NC01), *Y. pestis* (lane 3; gyrA-F01/gyrA-R03; restriction enzyme ApaI1) and *Y. pseudotuberculosis* (lane 4, gyrA-F01/gyrA-R03, restriction enzyme ApaI1). In lanes 3 and 4 of the original gel, a 73 bp fragment was just visible in addition to the fragments of 320 and 247 bp. However, this fragment could not be visualised on this photo prepared from the gel.

In addition, the primer pair gyrAF04/gyrA-R03 was also tested with 10 different strains of *Francisella tularensis* (cell lysates) as a source of DNA (Figure 6). The expected amplicon was produced in all cases and the DNA sequence of the products was determined. The complete sequence was similar to that found in literature, except for one base on a specific position, TTC, coding for the amino acid F, similar for all strains. The mutated triplet is TTT. However, the mutated base is at the third position of a codon and the mutated sequence still codes for the same amino acid sequence.

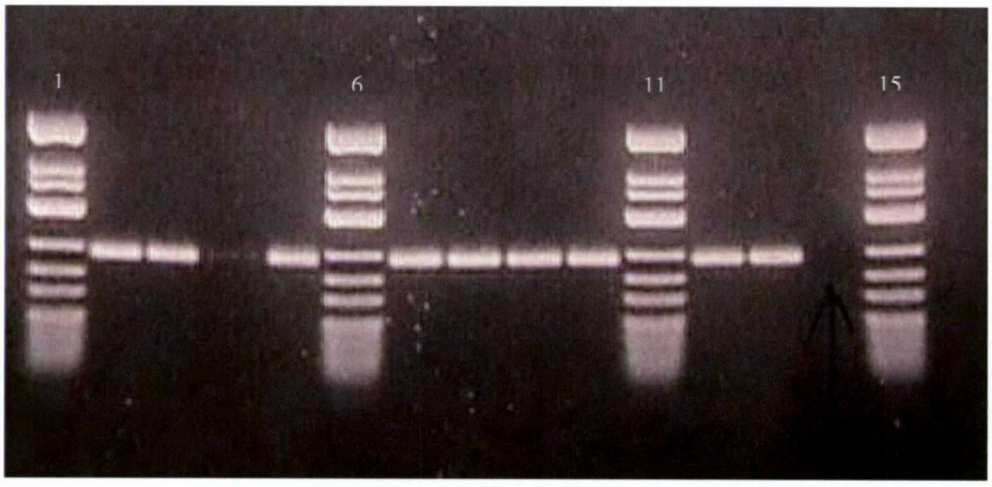

Figure 6 MW-marker is M9 (lanes 1, 6, 11 and 15). Primer used was gyrA-F04/gyrA-R03. The various strains of *Francisella tularensis* used were 021 (lane 2), 022 (lane 3),032, (lane 4), 040 (lane 5), 046 (lane 7), 080 (lane 8), 148 (lane 9), 155 (lane 10), 230 (lane 12) and 237 (lane 13). In lane 14, no DNA was added.

3.5 Selective amplification of the gyrA and the parC fragment in bacterial suspension of *E. coli*

A bacterial suspension of *E. coli* XL1 Blue was added to a PCR assay with the gyrA primer pair, gyrA-F01/R02, as well as to an assay with the parC primer pair, parC-F01/R01. The assay with the gyrA primer pair resulted in the production of one distinct amplicon of the gyrA fragment, 320 bp (Figure 7, lane 1). When using this primer pair, no parC fragment was found. The blanc assay, to which no DNA was added, did not result in the amplification of a product (Figure 7, lane 2).
The assay with the parC primer pair resulted also in the production of one specific amplicon, that of the parC fragment, 294 bp (Figure 7, lane 4). This time, no gyrA fragment was amplified. The blanc assay, to which no DNA was added, did not result in the amplification of a product (Figure 7, lane 5).

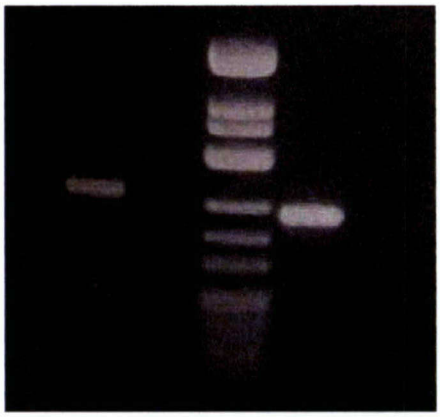

Figure 7 MW-marker is M9 (lane 3). DNA of a bacterial suspension of *E. coli* was amplified with the primer pairs gyrA-F01/gyrA-R02 (lane 1; blank in lane 2) and parC-F01/parC-R01 (lane 4, blank in lane 5).

Other experiments were performed in order to validate the PCR assays. Various *E. coli* strains were used as a source of DNA in the PCR assay. The primer pair for the amplification of a gyrA fragment used was gyrA-F01/gyrA-R02. In all assays, the expected product of 320 bp was found, indicating selective amplification of the gyrA fragments of the various strains (Figure 8, lanes 2-5). The amplified gyrA fragments of the various *E. coli* strains were sequenced (Table 1). In the case of *E. coli* XL1-Blue and JM109, the sequence of the various amplicons differed slightly from that found in literature for the wild type strain ATCC 11775. From the six point mutations that were observed, one mutation resulted in a mutation in the amino acid sequence (change from D to N). Furthermore, this mutation was on a position that is associated in literature with resistance against ciprofloxacin. Thus, our strains were mutated.

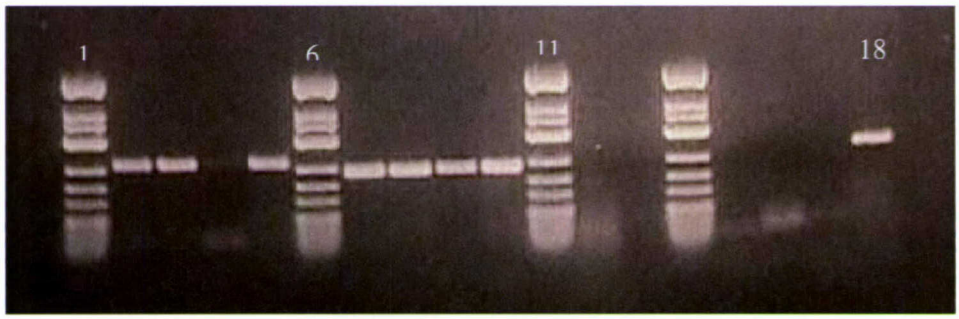

Figure 8 MW-marker is M9 (lanes 1, 6, 11 and 14). DNA of a bacterial suspension of various *E. coli* strains was used: XL1 Blue (lanes 2 and 7), JM109 (lanes 3 and 8) and the wild strain ATCC 11775 (lanes 5, 10 and 18). Amplification was performed with the primer pairs gyrA-F01/gyrA-R02 (lanes 2-5 and 15) and parC-F01/parC-R01 (lanes 7-10 and 16). No DNA was added in the PCR assay belonging to the lanes 15 and 16. Results from the lanes 4, 9, 12, 13 and 16 were not part of the underlying work, and are not described in this report.

Table 1 Point mutations in the QRDR region of the gyrA gene of two E. coli strains, compared to the wild type strain ATCC11775. Point mutations are marked yellow.

Strain	ATCC11775		XL1 blue		JM109	
Amino-acid position	Codon	Amino-acid	Codon	Amino-acid	Codon	Amino-acid
32	CGT	R	CG**C**	R	CGT	R
85	GTT	V	GT**C**	V	GT**C**	V
87	GAC	D	**A**AC	**N**	**A**AC	**N**
91	CGT	R	CG**C**	R	CG**C**	R
100	TAC	Y	TA**T**	Y	TA**T**	Y
111	TCC	S	TC**T**	S	TC**T**	S

The same *E. coli* strains were also used in a PCR assay with the primer pair parC-F01/parC-R01. This primer pair was used for the amplification of a parC fragment from the various strains. In all assays, the expected product of 292 bp was found, indicating selective amplification of the parC fragments of the various strains (Figure 8, lanes 7-10). The amplified parC fragments of the various *E. coli* strains were sequenced as well. In the sequence of the parC fragment of *E. coli* XL1-Blue and JM109, three point mutations were observed, in comparison to the sequence of the wild type strain ATCC 11775 that was found in literature. Three triplets, AGT, CAG and GGG were mutated into AG**C**, CA**A** and GG**C**, respectively. However, these point mutations were still coding for the same amino acids: S, Q and G, respectively (silent point mutation).

4 Discussion and conclusions

In case of exposure of military personnel to pathogenic bacteria, and BW agents in particular, rapid (same-day) reporting of susceptibility results may contribute to reduced mortality, morbidity and hospital costs, provided that results are immediately made available to the physicians in charge of the military personnel. Ultimately, this will result in a better management of diseased military personnel. Standard methods commonly used to detect susceptibility of bacteria to various antibiotics are laborious and will take up to two days. Therefore, other methods for the determination of the susceptibility profile of the bacteria involved are needed.

4.1 Antibiotic resistance related to the presence of specific genes (plasmids)

Resistance against antibiotic compounds can be due to the presence of specific genes (plasmids). Examples of this type of resistance are the antibiotics ampicillin and tetracycline. Ampicillin resistance can be associated with the presence of, for instance, the Ap-R gene, ß-lactamase in plasmids like pUC18, Bt-PA3 and pBR325. Tetracycline resistance is associated with the presence of, for instance, the Tc-R gene, tetracycline resistance protein in a plasmid such as pBR325. The presence of these genes, that are normally not present in the bacteria, can be detected with a PCR assay. The presence and expression of these genes confers resistance against tetracycline and ampicillin.

It was possible to design and develop three primer pairs that could be used to amplify the Ap-R and Tc-R genes from plasmids such as Bt-PA3, pUC18 and pBR325. Either as such, or from transformed whole cells. The genes associated with the antibiotic resistance could be detected. For the Ap-gene, the primer pairs were Ap-F04/Ap-R04, Ap-F05/Ap-R05, Ap-F06/Ap-R06 and 11031F014/11031R014. For the Tc-R gene these were Tc-F01/Tc-R01, Tc-F02/Tc-R02 and Tc-F03/Tc-R03. It was also possible to detect two different plasmids in one bacterium in two separate PCR assays. However, we did not perform any multiple PCR experiments with primer pairs for the amplification of the various genes from bacteria in one assay.

4.2 Antibiotic resistance related to specific mutations in essential genes of bacteria

Another type of resistance against antibiotic compounds is associated with specific mutations in essential genes of bacteria. This is the case for the military relevant ciprofloxacin, a fluoroquinolone, which is the first choice of therapy of anthrax infections. In the bacteria that were studied in this report, resistance appears mainly due to mutations in the intracellular targets of fluoroquinolones, the type II DNA topoisomerases gyrase and topoisomerase IV [Balas et al., 1998; Weigel et al., 1998]. Mutations in the quinolones resistant determining region (QRDR) of the parC gene, which encodes the A subunit of topoisomerase, and the gyrA gene, which encodes the A subunit of DNA gyrase are examples of mutations that are associated with most cases of resistance of the bacteria. It was found that homology of the QRDR region of various bacteria was rather high. To determine the extent of gyrA and parC mutations associated with the bacteria studied, sets of oligonucleotide primers were selected from conserved sequences in the flanking regions of the QRDR. Theoretically, these set of primers could be used to amplify and sequence fragments of the QRDR's from the bacteria like *Yersinia pestis*, *Francisella tularensis* and *Vibrio cholerae*.

4.3 The gyrA and parC genes of *Yersinia* species

It appeared possible to use the same primer pairs, gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03, for amplification of the gyrA gene in bacterial suspensions of *Yersinia pestis* and *Yersinia pseudotuberculosis*.

In view of the strong homology of the gyrA en parC of the Y. species, it would have been possible that the use of the gyrA primer pairs would also result in the amplification of the parC gene. If this were the case, sequencing of this mixture is not possible. But fortunately, only one product was amplified when using the gyrA primer pairs. This was determined by experiments in which amplified products were split into two distinct parts by restriction enzymes. The amplicon of 320 bp produced with the primer pair gyrA-F01/gyrA-R03 and *Y. pestis* and *Y. pseudotuberculosis* as a source of DNA, was digested in two smaller products of 247 and 73 bp after incubation with the restriction enzyme ApaI1 (Figure 5, lanes 3 and 4, respectively). In addition, some remainder of the 320 bp product was found. This might be due to the fact that the amplification product was not completely digested into two smaller products by the restriction enzyme. No other products could be visualized. This indicates that the primer pair gyrA-F01/gyrA-R03 was not able to amplify the parC fragment (292 bp) of the *Y.* species, and specifically amplifies gyrA. The formation of just one amplification product was also the result from another experiment in which the amplified gyrA fragments were sequenced. The DNA sequence of the fragments corresponded with the literature data on the sequences, indicating selective amplification of the gyrA fragment of the two species.

4.4 The gyrA and parC genes of *E. coli*

When *E. coli* was used as a source of DNA, the use of primer pair gyrA-F01/gyrA-R02 resulted in the production of the expected amplicon (Figure 4, lane 2). However, the primer pair gyrA-F01/gyrA-R03 was not able to amplify the gyrA fragment (Figure 4, lane 3), indicating that the sequence of the gyrA fragment in this organism diverge from the conserved sequences shared by the *Y.* species. The amplicon of 320 bp produced with the primer pair gyrA-F01/gyrA-R02 and *E. coli* as a source of DNA, was divided in two smaller products of 180 and 140 bp after incubation with the restriction enzyme NC01 (Figure 5, lane 2). In addition, some remainder of the 320 bp product was found. No other products could be visualized. Again, it is most likely that in the original assay one amplification product was formed which was not completely divided into two smaller product by the restriction enzyme. Therefore, the product in a sample from the same PCR assay that was not incubated with restriction enzyme was sequenced. Only one product was found and its sequence corresponded very well with the sequence of the gyrA fragment as found in literature, indicating selective amplification of the gyrA fragment and not the parC fragment of *E. coli*.

However, the sequence of the various amplicons when using *E. coli* XL1-Blue and JM109 as a source of DNA, differed slightly from that found in literature for the wild type strain ATCC 11775. From the six point mutations that were observed, one mutation resulted in a mutation in the amino acid sequence. Furthermore, this mutation was on a position that is associated in literature with resistance against ciprofloxacin (QRDR). Thus, the strains we have available in our laboratory were mutated. One in a position that is associated with resistance against ciprofloxacin. It is essential to compare this mutation with the actual behaviour of these strains when cultured on

ciprofloxacin containing media. This work was performed in a subsequent study which is reported in Report II. Shortly, it was found that the two mutated strains of *E. coli*, XL1-Blue and JM109, are still susceptible to ciprofloxacin. Although position 87 of gyrA is known to be involved in ciprofloxacin resistance, the change from D to N is not dramatic, in the sense that the amino acids D and N are quite similar in structure and nature. This would explain why this point mutation in *E. coli* strains XL1-blue and JM109 does not result in resistance [Broekhuijsen and Van Dijk, 2005].

4.5 The gyrA and parC genes of *Francisella tularensis*

Based on the sequence of the conserved sequences flanking the QRDR of *F. tularensis*, it was anticipated that the primer pairs gyrA-F01/gyrA-R02 and gyrA-F01/gyrA-R03 would not be able to amplify the gyrA fragment from this species. Therefore, another primer pair was developed, gyrA-F04/gyrA-R03. This primer pair appeared to be able to produce the expected amplicon with a lower (Figure 4, lane 11) or a higher amount (Figure 4, lane 10) of DNA in the PCR assay.
In order to validate the PCR assay, the amplified gyrA fragment of *F. tularensis* 237 was sequenced. The DNA sequence of this fragment corresponded with the literature data on the sequences, indicating selective amplification of the gyrA fragments of the various species.
In addition, the same primer pair was also tested with 10 other, different strains of *Francisella tularensis* as a source of DNA (Figure 6). The expected amplicon was produced in all cases and the DNA sequence of the products was determined. The complete sequence was similar to that found in literature, except for one specific silent point mutation, in all strains.

4.6 Conclusions

From the results obtained in this feasibility study, it can be concluded that it is possible to detect specific plasmid genes associated with resistance to certain antibiotics in whole bacteria. In addition, it appeared possible to set up methods to detect mutations in essential genes of bacteria. The pattern of the mutations can provide information on the possibility of antibiotic resistance against military relevant antibiotics.

One must keep in mind that detection of the presence of a gene or the detection of the mutation in a gene only indicates the possibility of the development of resistance against antibiotics. It might be well possible that bacteria can be susceptible to a certain type of antibiotic, in spite of the presence of a gene associated with resistance against that antibiotic compound. Only phenotypic methods providing susceptibility results after 4 –24 hours will give an unequivocal answer to whether a pathogen is antibiotic resistant or not. But, the use of the PCR results can speed up the start of an efficient therapy. In addition, it is also possible that the use of the antibiotic compound in a person with demonstrated presence of (mutated) genes associated with resistance without actual resistance, will result in the development of resistance against that compound in the future. Therefore, it is better, if possible, to avoid the use of antibiotic compounds for which (mutated) genes associated with resistance are present.

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6 Signature

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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

During their duties, military personnel can be exposed to military relevant agents. In order to start a rapid and efficient therapy, especially in the case of micro-organisms that are resistant to (several) antibiotics, the identity of both the agent and the genes associated with the resistance against antibiotics must be known. A method that provides rapid and unequivocal information on the presence of specific genes is essential. Polymerase Chain Reaction (PCR) is such a method. The results obtained in this report suggest that it is possible to detect specific genes that are related to resistance against antibiotics such as tetracycline and ampicillin. It is also possible to detect mutations in essential genes of bacteria that are related to resistance against military relevant antibiotics such as ciprofloxacin. Detection of such genes with PCR or detection of mutations by means of PCR, followed by DNA sequencing can help to construct the outline of an efficient therapy. This will contribute to fulfilling one of the main objectives of the Ministry of Defense in the area of 'Passive NBC Protection', i.e. 'Protection of personnel against adverse health effects as a result of (non intentional) exposure to biological agents under operational circumstances'.

16. DESCRIPTORS	IDENTIFIERS
Ciprofloxacin	Antibiotic resistance
Tetracycline	Screening
Ampicillin	PCR

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11	TNO Defensie en Veiligheid, vestiging Rijswijk, Manager Business Unit BC Bescherming (kennis), dr. R.W. Busker
12/13	TNO Defensie en Veiligheid, vestiging Rijswijk, Informatie- en Documentatiedienst
14/16	TNO Defensie en Veiligheid, vestiging Rijswijk, Business Unit BC Bescherming, A. Tigchelhoff, Dr. M. Polhuijs en A.M.B.C. de Vries
17/19	Bibliotheek KMA
20	TNO Defensie en Veiligheid, vestiging Rijswijk, Marketing en Communicatie, digitale versie via Archief
21	TNO Defensie en Veiligheid, vestiging Rijswijk, afdeling Tekstverwerking (digitale versie)